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PRINCIPAL INVESTIGATOR: Chung S. Park, Ph.D.

CONTRACTING ORGANIZATION: North Dakota State University
Fargo, North Dakota 58105

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13. ABSTRACT (Maximum 200) Dietary deficiency of lipotropes is known to be carcinogenic in itself. This study examined 1) the susceptibility of female rats previously exposed to lipotrope-modified diets to nitrosomethylurea (NMU)-induced mammary carcinogenesis; and 2) how lipotrope-modified diets modulate DNA methylation and gene expression in mammary tissue. Female rats (36 d of age) were assigned to one of the following groups: control-synthetic diet (CSD), containing all required lipotropes; methyl-deficient diet (MDD), lacking all lipotropes; and methyl-additive diet (MAD), containing 1.5-fold the amount of each lipotrope as in CSD. Rats were injected with NMU after a 2-wk dietary treatment period, and 2 d after NMU administration all treatment groups were fed CSD for the remaining experimental period. Mammary tissues were collected from rats just prior to NMU administration. The MDD group had the shortest latency period. The number of tumors and tumor volume were greatest in the MDD rats. Dietary modification of lipotropes altered the DNA methylation pattern of ornithine decarboxylase gene. DNA was significantly hypomethylated in mammary tissues of the MDD rats. These results suggest that dietary deficiency of lipotropes led to changes in DNA methylation and enhanced NMU-induced mammary carcinogenesis.				
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A. INTRODUCTION:

1. Background

Lipotropes (methionine, choline, folic acid, and vitamin B₁₂) interact extensively in the supply and regulation of intercellular pools of methyl groups. They are essential for the synthesis and methylation of DNA, the production of nucleoproteins and membranes, and the metabolism of lipids. Lipotrope deficiency, or methyl deficiency, enhances some forms of chemically-induced hepatocarcinogenesis (1). Further, dietary deficiencies of choline and methionine have carcinogenic effects by themselves (2, 3). Most research on lipotropes has concentrated on liver abnormalities. However, lipotrope deficiency affects almost every organ in the body, particularly when animals are in a state of high metabolic activity and growth (4), and much of the data on the relationship between lipotrope deficiency and mammary carcinogenesis was derived from male rats. The mechanism underlying the enhanced mammary tumorigenesis by lipotrope deficiency is unclear. Very little is known about the effect of dietary lipotrope supplementation on tumorigenesis, and these findings are not consistent (5, 6).

2. Purpose

We hypothesize that lipotrope (choline, methionine, folic acid, and vitamin B₁₂)-modified diets alter normal DNA methylation. The genomic DNA methylation may be related to the lipotrope-mediated expression of the transcription factors, *fos*, *jun*, and thus the activation of the transcription regulatory complex, AP-1. This activated or repressed AP-1 regulates expression of genes (e.g. ornithine decarboxylase) which are responsible for cell proliferation and differentiation. Significance of this research endeavor is to contribute to the understanding of the role of dietary lipotropes in the initiation and prevention of chemically-induced mammary carcinogenesis in the female rat.

The overall goal of the proposed research is to develop a better understanding of the role of dietary lipotropes in mammary gene expression and carcinogenesis.

Specific aims are:

- 1) to determine the extent to which dietary lipotropes alter genomic DNA methylation levels and patterns and the expressions of genes (*jun* and ornithine decarboxylase) involved in the proliferation of mammary cells.
- 2) to determine the susceptibility of female rats previously exposed to lipotrope-modified diets to NMU-induced mammary carcinogenesis.

3. Scope of Study

The focus of the present experiment was to determine the susceptibility of female rats previously exposed to lipotrope-modified diets to mammary carcinogenesis induced by a chemical carcinogen, nitrosomethylurea (NMU).

This experiment was composed of two feeding phases. Phase I was a 2-week treatment period (from 5 to 7 weeks of age) to lipotrope-modified diets (control, methyl-deficient, and methyl-additive) prior to NMU administration. The focus of Phase I was on changes in DNA methylation and expression of genes (*jun* and ODC) involved in mammary cell proliferation and tumorigenesis (Specific Aim 1). Phase II, the period after NMU injection, mainly was concerned with the incidence of mammary tumors (Specific Aim 2).

B. BODY OF REPORT:

1. Experimental Methods

Animals and Experimental Diets. One hundred thirty-two (132) weanling female Sprague-Dawley rats (26 days of age) were housed individually in wire mesh-bottom cages and acclimated to the experimental environment of 25°C and 50% relative humidity with a 12-hr light/dark cycle for 10 days. Rats were offered control-synthetic diet (CSD) (ad libitum) during this 10-day acclimation period. The rats were randomly assigned to one of three dietary groups: 62 rats for CSD and 35 rats each for methyl-additive diet (MAD) and methyl-deficient diet (MDD) groups. Just prior to the start of the three dietary treatments (age=36 days), a random sample from the MAD (n=5), CSD (n=7), and MDD (n=5) groups was sacrificed. This provided initial baseline data. The remaining rats (n=30 for MAD and MDD and n=55 for CSD) were offered their respective assigned diets ad libitum for 14 days. At the end of the 14-day period (50 days of age) of dietary treatment, five rats were sacrificed from each of the three dietary treatment groups. At 50 days of age all of the rats in the MAD and MDD groups were injected with NMU, and half of the rats in the CSD group were NMU injected while the other half was injected with a placebo of 0.9% NaCl. Two days after NMU administration all treatment groups (age=52 days) were fed CSD with ad libitum access for the remaining duration of the experiment. Mammary tissues were collected just prior to NMU administration.

The basal diet, lipotrope-deficient diet (LDD), was an amino acid-defined semisynthetic formulation (Harlan Teklad, TD 90045, Madison, WI) lacking choline, methionine, folic acid, and vitamin B₁₂ (Table 1). The CSD was LDD supplemented with 5 g/kg DL-methionine, 2 g/kg choline chloride, 5 mg/kg folic acid, and 100 µg/kg vitamin B₁₂. The MDD was LDD supplemented with 9 g/kg DL-homocysteine. The MAD was LDD supplemented with 1.5-fold the amount of each lipotrope as in CSD. Rats were offered free access to diets, and body weights were recorded at weekly intervals.

Mammary Tumor Induction and Measurements. At 50 d of age, 25 rats from each treatment group were injected s.c. with 50 mg of NMU per kg of body weight (7). The NMU (10 mg/ml) was prepared by dissolving in acidified 0.9% NaCl solution (8). Two d after NMU treatment, all rats were placed on the CSD for the remaining experimental period. At weekly intervals, beginning 4 wk after NMU injection, rats were palpated for mammary tumors. Upon detection, the position and date of appearance of mammary tumors were recorded. Twelve wk after NMU injection, mammary tumor sizes were determined by Vernier caliper measurements of the subcutaneous mass, and tumor volumes were calculated as described previously (8, 9).

Mammary Tissue Collection. Mammary tissue was collected from 5 rats from each dietary group just prior to NMU administration (age=50 d). Mammary tissues were frozen immediately in liquid nitrogen and stored at -70°C until analysis.

TABLE 1. Composition of the Amino Acid-defined Basal Diet (TD 90045)

Ingredient	Amount	Ingredient	Amount
	g/kg		g/kg
Sucrose	407.4	Calcium pantothenate	0.066
Corn starch	250.0	Ψ-Inositol	0.11
Corn oil	50.0	Manadione	0.0496
Beef tallow	50.0	Nicotinic acid	0.099
Cellulose	30.0	Pyridoxine HCl	0.022
Calcium phosphate, dibasic	4.5	Riboflavin	0.022
L-Alanine	3.5	Thiamine HCl	0.022
L-Arginine HCl	12.1	Glycine	23.3
L-Asparagine	6.0	L-histidine HCl.H ₂ O	4.5
L-Aspartic acid	3.5	L-isoleucine	8.2
L-Cystine	3.5	L-leucine	11.1
L-Glutamic acid	40.0	L-lysine HCl	18.0
Mineral mix, AIN-76*	35.0	L-phenylalanine	7.5
Ethoxyquin (antioxidant)	0.02	L-proline	3.5
Dry tocopherol acetate (500 U/g)	0.24	L-serine	3.5
Dry retinoic palmitate (500,000 U/g)	0.0396	L-threonine	8.2
Dry cholecalciferol (500,000 U/g)	0.0044	L-tryptophan	1.8
Ascorbic acid, coated (97.5%)	1.0	L-tryosine	5.0
Biotin	0.0004	L-valine	8.2

*Harlan Teklad No. TD-170915, Madison, WI; in g/kg of diet: CaHPO₄, 500.0; NaCl, 74.0; Potassium citrate, monohydrate, 220.0; K₂SO₄, 52.0; MgO, 24.0; Manganous carbonate, 3.5; Ferric citrate, USP (16.7% Fe), 6.0; Zinc carbonate, 1.6; Cupric carbonate, 0.3; KIO₃, 0.01; Na₂SeO₃.5H₂O, 0.01; CrK(SO₄)₂.12H₂O, 0.55; Sucrose, finely powdered, 118.03.

DNA Methylation Levels by DNA Methyltransferase Reaction. DNA methyltransferase (Mtase) was prepared from Friend erythroleukemia cells (FLC; donated by Dr. J.L. Hoffman, University of Louisville, Louisville, KY), by using the procedures described by Wainfan et al. (10). The enzyme reaction, which catalyzes the transfer of methyl groups from S-adenosylmethionine (AdoMet) to DNA, was carried out under the condition of enzyme excess, so that the extent of methyl group incorporation into DNA depended upon the type and concentration of DNA and was unaffected by increasing enzyme concentration. The method described by Wainfan et al. (10) was used as the basis for the assay. Each assay tube contained 2 μ g of DNA and DNA Mtase at ≥ 1 unit/ μ g DNA in 200 μ l final volume, with 100 mmol/L imidazole (pH 7.5), 20 mmol/L EDTA, 0.5 mmol/L DTT, and 16 μ mol/L S-[methyl- 3 H] AdoMet (10-20 μ Ci mmol/L). One unit of Mtase transfers 1 pmol/L of methyl groups in 15 min to a standard preparation of DNA from FLC grown in the presence of L-ethionine (11). Reactions were terminated by adding sarkosyl to 0.6%, followed by a 20 min incubation at 37°C with 40 μ g/ml of proteinase K. Two volumes of 0.5 mol/L NaOH were added, and incubation continued at 60°C for 10 min. After cooling on ice, DNA was precipitated by adding 1/6 volume of 5 mol/L perchloric acid. After 15 min, the precipitate was washed into GF/B filters (Whatman, Hillsboro, OR) and rinsed 3 times with ice-cold 6% perchloric acid and twice with ice-cold 95% ethanol. Radiolabel on dried filters was counted (LS 5801, Beckman Instruments, Inc., Fullerton, CA).

RNA Extraction and Northern Blot Analysis. Total RNA was extracted from frozen mammary tissues by the guanidine thiocyanate and phenol extraction method (12). Poly(A+) RNA was isolated from pooled total RNA using oligo dT cellulose columns (5 Prime \rightarrow 3 Prime, Inc., Boulder, CO). Poly(A+) RNA (5 μ g per lane) was fractionated by electrophoresis on a 1% agarose gel containing 2.2 mol/L formaldehyde and transferred to a nylon membrane. The membrane was baked for 1 h at 80°C in a vacuum oven and hybridized with cDNA probe, ornithine decarboxylase (ODC) (donated by Dr. P.J. Blackshear, Duke University, Durham, NC). The denatured cDNA probes were labeled with [32 P] dATP by random priming method (Muliprime DNA Labeling System, Amersham Life Science, Arlington Heights, IL). The membrane was prehybridized for 3 h and hybridized for 17 h, respectively, at 42°C. After washing, the membrane was exposed to X-ray film (Kodak, Rochester, NY) with an intensifying screen at -70°C. The signals on Northern blots were quantitated with the Personal Densitometer SI System (Molecular Dynamics, Sunnyvale, CA). The blots were rehybridized with glyceraldehyde-3-phosphate dehydrogenase cDNA (American Type Culture Collection, Rockville, MD) as the control probe.

DNA Methylation Patterns by Southern Blotting. Frozen mammary tissue was pulverized in liquid N₂ and homogenized in 10 mmol/L Tris-HCl buffer containing 25 mmol/L EDTA, 100 mmol/L NaCl, 0.5% SDS, and 0.01 mg/ml proteinase K. Following an 18 h incubation at 50°C, the homogenate was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), followed by an extraction with chloroform:isoamyl alcohol (24:1). The DNA was precipitated in ethanol at -70°C for at least 1 h, spooled out with a plastic purification rod, and dissolved in sterile distilled water (13). The DNA was treated with RNase (0.5 μ g/ μ L) for 1 h at 37°C, re-extracted in phenol:chloroform:isoamyl alcohol, reprecipitated in ethanol, and dissolved in sterile distilled water. Genomic DNA was digested with HpaII or MspI restriction endonucleases (New England Biolabs, Beverly, MA) at 37°C for 20 h according to the assay conditions recommended by the supplier. The

completeness of digestion was monitored by electrophoresis with 1 μ l of digested mix. Digested genomic DNA (10 μ g/lane) was separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane. The probe, ODC was radiolabeled with [32 P]dATP by random priming method (Multiprime DNA Labeling System, Amersham Life Science, Arlington Heights, IL). The nylon membrane was prehybridized for 3 h and hybridized for 17 h, respectively, at 42°C. Hybridization signals were visualized by autoradiography.

Statistical Analyses. In vitro methylation data were analyzed by the general linear model procedures (14). Comparisons of cancer latency with the tumorigenesis data were based on cancer-free times using the Mantel-Haenszel life table (15). Differences in tumor numbers were evaluated by analysis of variance following square root transformation (15). Statistical comparison of tumor volume was conducted with the nonparametric Kruskal-Wallis test (16).

2. Results

Tumor Incidence, Volume, and Latency Period. The tumor incidence was lower in the MAD group than in the CSD and MDD groups at week 12 after NMU injection. The latency period was shortest in the MDD group (79.4 d; $p > 0.05$) (Table 2). The number of tumors per rat was significantly ($p < 0.05$) higher in the MDD rats compared with those in either the CSD or MAD group. The tumor volume in the MDD group was also greater ($p < 0.05$) than that in other dietary treatment groups.

TABLE 2. Mammary Tumor Incidence, Latency Period, Numbers, and Volume in Rats Fed Lipotrope-modified Diets*

	Treatment**			SEM
	CSD	MDD	MAD	
Tumor incidence (%) [#]	64	68	48	• •
Latency period (day) ^{##}	83.5	79.4	85.2	4.6
No. of tumors [†] (tumors/rat)	1.2 ^a	2.0 ^b	1.3 ^a	0.3
Tumor volume [†] (cm ³ /rat)	3.2 ^a	13.9 ^b	3.3 ^a	2.7

*Values are means, n=25.

**CSD = control synthetic diet; MDD = methyl-deficient diet; MAD = methyl-additive diet.

[#]Percentage of animals that developed at least one tumor by 12 wk after NMU administration.

^{##}Determined as the days between NMU injection and the appearance of the first tumor.

[†]Values were determined at 12 wk after NMU administration.

^{a,b}Means in rows with different superscripts differ ($p < 0.05$).

DNA Methylation Levels and Patterns. The DNA was hypomethylated ($p < 0.05$) in the MDD group (Table 3). However, the methylation levels between CSD and MAD groups were not different. Changes in the methylation pattern of the ornithine decarboxylase (ODC) gene were detected in mammary tissues of the MDD group after 2 wk of dietary treatment (lane 5) [Figure 1].

TABLE 3. In Vitro Methylation of Mammary DNA from Female Rats Fed Lipotrope-modified Diets*

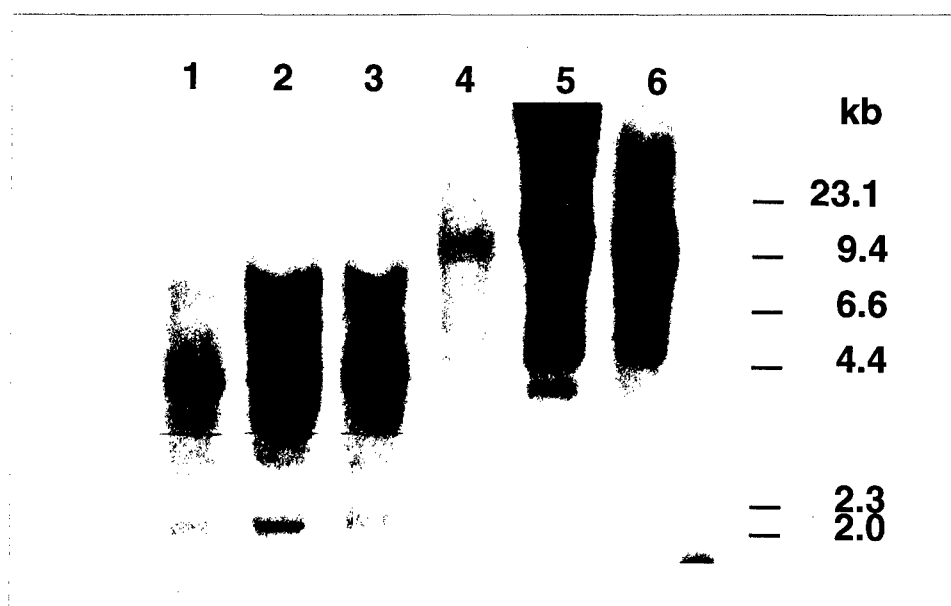
Treatment**			SEM
CSD	MDD	MAD	
Cpm/2 μg x 10 ⁻³			
9.0 ^a	18.5 ^b	8.5 ^a	0.6

*Values are means \pm SEM of 4 determinations and are expressed as the amount of incorporation of methyl groups into DNA.

**CSD = Control-synthetic diet; MDD = Methyl-deficient diet; MAD = methyl-additive diet.

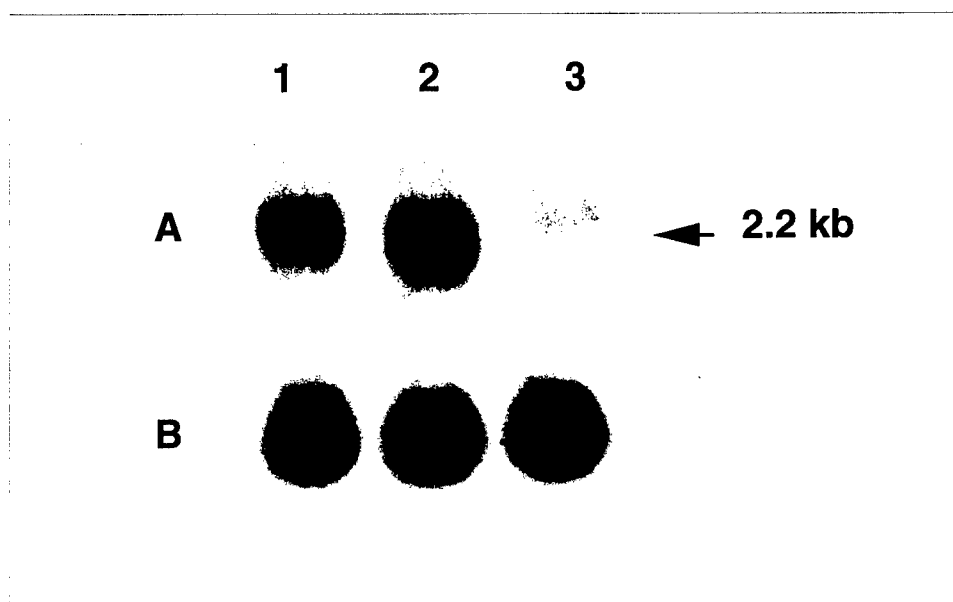
^{a,b}Values with different letters are significant ($p < 0.05$).

FIG. 1. Methylation patterns of the ornithine decarboxylase gene in rat mammary tissues. Southern blot analysis of HpaII- or MspI-digested genomic DNA (10 μg) from mammary tissues of female rats fed lipotrope-modified diets for 2 wk. The resulting DNA fragments were separated by 1% agarose gel electrophoresis and transferred to a nylon membrane. The membrane was hybridized to a 2.16 kb EcoRI fragment of pODC-2. Size standards include HindIII digested lambda DNA. Lanes 1-3, MspI digested DNA; lanes 4-6, HpaII-digested DNA. Lanes 1 and 4, CSD, control-synthetic diet; lanes 2 and 5, MDD, methyl-deficient diet; lanes 3 and 6, MAD, methyl-additive diet.



Gene Expression. The level of ODC mRNA transcript was 3.7- and 2.8-fold greater in the MDD and CSD groups, respectively, than in that of the MAD group (Figure 2).

FIG. 2. Effect of lipotrope-modified diets on levels of ornithine decarboxylase mRNA in rat mammary tissues (A). Northern blot analysis of pooled (n=5) poly(A⁺) RNA (5 µg/lane) from mammary tissues of female rats fed lipotrope-modified diets for 2 wk. The RNA was fractionated on a 1% agarose gel and transferred to a nylon membrane and then hybridized to a 2.16 kb EcoRI fragment of pODC-2. Lane 1, CSD, control-synthetic diet; lane 2, MDD, methyl-deficient diet; lane 3, MAD, methyl-additive diet. The membrane was reprobbed with glyceraldehyde-3-phosphate dehydrogenase cDNA as a control (B).



3. Discussion

DNA methylation has been viewed as a long-term inactivating or inhibiting modulator that could play a decisive role in the regulation of gene activity or tumorigenesis. The DNA was hypomethylated in mammary tissue from the MDD group and this may explain the increased carcinogenesis in these rats. The loss of methylation occurs primarily as a result of failure to methylate newly synthesized DNA after replication. This disruption of normal DNA methylation has been implicated in the development of tumors. The lipotrope-deficient diet depressed normal methylation of DNA by lowering the pool of AdoMet. When the cell prepares for normal division, methylation of the daughter strands of DNA may be diminished because of insufficient AdoMet. The strand may have less 5'-methylcytosine (5mC) than the parent DNA. Because 5mC is regarded as having a role in inhibiting expression of genes, hypomethylation of DNA would be expected to

increase the number of genes capable of being expressed. In the methylation pattern of the ODC gene, a 3.4 kb band clearly seen in digests of DNA from the MDD group suggested that the site is unmethylated. This fragment was barely observed in the HpaII digests of DNA from the CSD and MAD groups. This alteration in the DNA methylation pattern may be an important factor in the progression of cancer in chemically-induced mammary cells.

The induction of the ODC gene is closely correlated with cell proliferation and differentiation and has been deemed a marker of tumor promotion (17). As evidenced by an increase in ODC gene expression in mammary tissues of the MDD rats, hypomethylation could play a role in cell transformation (18, 19). The transcriptional activity of the ODC gene is increased in transformed cells (20). Hypomethylation of the ODC gene in the MDD group suggests an increase in transcriptional activity. The increase of ODC gene expression may be critical for cell transformation (21), and therefore, for the progression of cancer in chemically-induced mammary cells. In an NMU-induced mammary tumor study, Manni et al. (22) demonstrated that ODC over expression is associated with breast cancer progression via altering the polyamine metabolic pathway.

Our data showed that induction of the ODC gene is sensitive to dietary depletion of methyl group-containing nutrients. Inhibitors of ODC suppress tumor promotion (23). Mammary tumor growth was significant in the MDD group as manifested by the latency period, the number of tumors per rat and the total tumor volume compared with the other groups. Therefore, changes in DNA methylation may be an underlying cause for the effects of methyl-deficient diet on mammary tumor initiation.

The MAD group had low tumor incidence during the early phase of study, but excess methyl groups in the diet did not affect the DNA methylation level and pattern. However, rats fed MAD for 2 wk had less gene expression of ODC than the other groups. Although the level of total genomic DNA methylation was not different between the CSD and MAD groups, the extent of methylation in the regulatory regions of the ODC gene might be increased in the MAD group. The relevance of gene expression and methylation in the promoter region of ODC gene to dietary lipotropes remains to be established. Since DNA methylation can repress transcription, the presence of excess methyl group in MAD may lead to gene repression of ODC.

4. Notes

Rat growth data and *jun* Southern analysis (presented in the previous reports) are not included in the final report because they bear little or no biological significance to this study.

Also, the gene expressions of *fos* and *Ha-ras*, ODC enzyme activity, and AP-1 gel shift assay are not reported due to procedural and technical difficulties.

C. CONCLUSIONS:

Dietary deficiency of lipotropes significantly enhanced NMU-induced mammary carcinogenesis in female rats. DNA methylation of the ODC gene was altered in mammary tissues of

rats fed the lipotrope deficient diet. It appeared that DNA methylation may regulate the expression of certain genes involved in cell proliferation and differentiation thereby affecting the initiation and progression of mammary tumorigenesis. Although human diets are not normally lipotrope deficient, an excess intake of alcohol, administration of certain therapeutic drugs, or chemical carcinogens are capable of leading to the inhibition of DNA methylation either by reducing AdoMet levels or by inactivating DNA methyltransferase (24, 25).

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Personnel (not salaries) receiving pay from this effort:

Danielson, Christen; undergraduate student